OBSERVATIONS AND TAXONOMIC CONSIDERATIONS ON A *TETRASPORIDIUM* (CHLOROPHYTA, TETRASPORALES) FOUND IN PORTUGAL

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ABSTRACT - *Tetrasporidium javanicum*, an infrequent Tetrasporales, is cited for two localities in Portugal, one in the center and the other in the north of this country, and its morphological features are described. Its cytological variability is discussed and, as a result, *T. fottii* is considered untenable as an independent species. Some parameters of the habitat are also provided. This is, so far, the westernmost report of this organism, and the first one for Portugal.

RÉSUMÉ - *Tetrasporidium javanicum*, Tetrasporales peu fréquente, est signalé dans deux localités du Portugal, l'une au centre et l'autre au nord de ce pays. La morphologie de cet organisme est décrite et sa variabilité cytologique est discutée. D'après cette discussion, on considère que *T. fottii* ne doit pas être retenu comme une espèce indépendante. Des paramètres physico-chimiques de son habitat sont aussi présentés. Il s'agit, à notre connaissance, de la mention la plus occidentale de cette algue.

KEY WORDS : phytogeography, taxonomy, Tetrasporales, *Tetrasporidium fottii*, *T. javanicum*.

INTRODUCTION

In April 1991 one of our students (Ângelo E.R. Ferreira) brought into the laboratory a sample of algae he had collected in a small stream near his house in Passadouro, Aguada de Baixo (Águeda, central Portugal). It was mainly composed of *Batrachospermum* and a mucilaginous green thallus in which perforations could be seen even with the unaided eye. Its cells showed tetrasporalean characteristics but the absence of pseudocilia (verified with oil immersion objectives in phase-contrast) precluded the possibility of it being a *Tetraspora*. A consultation of Bourrelly's (1981) first volume of "Les Algues d'eau douce" yielded its identification as *Tetrasporidium javanicum* Möbius. The organism was then regularly collected for about a month after which it completely disappeared from the stream. Quite curiously, the very same alga was collected by the very same student from a locality in a mountain in the north of Portugal (Gerês) during a weekend visit in May.

Since it was first described in 1893, from a locality in Java, this organism has not been reported many times, and mostly for tropical and sub-tropical regions (Iyengar, 1932; Sarma & Suryanarayana, 1969; Nurul Islam, 1970; Pandey *et al.*, 1980). Only twice has the genus *Tetrasporidium* been reported for localities in Europe, the first time for Czechoslovakia by Fott *et al.* (1965), and the second one for ponds in Brittany (France) by Couté & Tracana (1981). In spite of the relatively few occasions in which it was described, when it comes to details in morphological and cytological characteristics a number of differences can be found between the observations of the several authors, which brings about some confusion as to the correct identity of the organism, as well as to its rightful systematic position.

MATERIALS AND METHODS

The observations were made on the living material, collected from the stream at Passadouro (where it grew attached mainly to *Callitriche stagnalis* Scopoli), as well as from Gerês (where it was found in a small ditch along a road). The organism was also grown for some time in "liquid Chlorella" (LCol) culture solution (for composition see Santos & Mesquita, 1986) at room conditions, but no attempt was made to isolate it.

A WILD M20 microscope equipped with a camera lucida was used for the drawings, and a DIALUX 20EB (equipped with a 1.3 NA oil immersion objective) was used for the photographs. Some physico-chemical parameters of the water in the stream at Passadouro were determined the seventh of May, when the colonization by *Tetrasporidium* was quite extensive. Conductivity, pH, water temperature, and dissolved oxygen were measured *in situ*, and the other analyses were performed after the arrival at the laboratory, by current methods (Rodier, 1971; Apha, 1971).

OBSERVATIONS AND RESULTS

Description of the organism (based mainly on our own observations)

Macroscopically the alga formed soft bright green masses attached by one end to several substrata[1] (mainly to the branches of *Callitriche stagnalis*) and stretched by the flowing water, which made them look rather narrow and reach a maximum length of ca. 10cm (fig. 8).

The adult thallus, consisting of a pouch-like mucilaginous layer with numerous perforations, appears under the microscope as a flattened bag in which the two layers anastomose quite frequently, giving it a net-like appearance when observed in low magnification (fig. 9). The cells lie within the mucilage in approximately one layer without any wall or cytoplasmic formation connecting them (fig. 10). They are ellipsoid to almost spherical (ranging from 6 to 10-(11) μm in length) and show a green parietal chloroplast with a four to six lobed margin and a large pyrenoid (ca. 1 μm to over 3 μm in the larger cells); the pyrenoid is located in the middle enlarged part of the chloroplast and is sheathed by starch grains, this enclosure frequently being composed of two distinct halves (figs. 1 and 13A). Two contractile vacuoles are usually present near the anterior part of the cell, where a small round papilla can sometimes be seen. In the anterior or in the medium portion of the chloroplast an eyespot is sometimes present. The interfase nucleus, showing a large nucleolus, is often visible without co-

[1] In the stream at Passadouro, besides *Callitriche stagnalis*, it was found attached to other aquatic plants, small stones, and even, in large numbers, to a plastic tube. In the ditch in Gerês it was found on small branches and leaves fallen from nearby trees.

loration and can be found in the posterior, medium, or anterior part of the cell. Numerous small granules can be seen in the cytoplasm. The cell wall is usually thin, although in some cells it may reach over 0.5 μm in thickness. Cells of any size can, under certain circumstances, produce two flagella of equal length (fig. 2), which can remain immobile or gain motility, in which case these cells swim away from the mucilage, thus leaving the thallus. The cells multiply themselves through the division of the contents of a cell into 2-8 autospores (figs. 3, 10, 11 and 12); these daughter cells then slowly separate from each other as the mother enlarges without breaking up until it eventually gelatinizes completely. Propagation of the organism occurs through the fragmentation of the thallus and also through the formation and release of biflagellate cells that swim about, attach themselves to a substrate, and produce a new thallus. The young thallus is a compact mass of mucilage of cylindrical or clavate shape, attached by one end, with the cells scattered below its surface.

The size of the cells in culture was found to be more variable; in a 6 day old culture cells could be found that reached 13 μm in diameter, and in a very unhealthy looking thallus of one month old culture, contamined by a large amount of a *Stigeoclonium* species, many of the cells were extremely reduced (down to a minimum of 3.5 μm) and some very large cells of about 17 μm could be seen.

The position of the chloroplast (and, of course, of the pyrenoid) is also variable, even in field material. In many cells, especially in small ones (and, as might be expected, in most autospores), the pyrenoid occupies a lateral position and the chloroplast does not fill the posterior end of the cell. In other cells the pyrenoid is near or at the rear portion of the cell and, in such cases, the chloroplast may seem in optical section to be cup-shaped; it is not, however, a real cup-shaped chloroplast like the one in, e.g., *Chlamydomonas reinhardtii* Dangeard, as the observation of the cell near its surface (where the lobes of the chloroplast are visible) always demonstrates (fig. 4 and 13).

Two contractile vacuoles pulsating in the anterior part of the cell seem to represent its "normal" state in the natural environment, since in all freshly collected thalluses examined they were evident in most of the cells. Only in situations in which the organism was submitted to some kind of stress the contractile vacuoles were found to be lacking in most cells; this was the case in microscope slides left overnight in the microscope, in which only the mucilage prevented total drought, and in old contaminated cultures.

The eyespot was present in all flagellated cells examined and also in non-flagellated cells; the proportion of the latter that showed an eyespot was, however, quite different in different collections, and varied, presumably, with the physiological state of the cells. In the first collected thalluses most of the cells (including autospores) had an eyespot, whereas in later collections few cells were found exhibiting one. In a culture a week old, in which there was a large density of cells in the mucilage, and many cells were sporulating, not one cell was found with an eyespot, although in all of them contractile vacuoles could be seen.

The numbers of autospores most frequently produced were 4 and 8. In the former case the 4 daughter cells can be formed simultaneously (fig. 5), or there can be a first division into two cells, which undergo a second, not always synchronous, division so that groups of three cells can be found in which one cell is bigger than the other two (fig. 6). The formation of two consecutive generations of autospores inside the still clearly visible parental cell wall was observed in culture. The formation of motile flagella by recently divided cells (lying very close to each other and to the mother cell wall) has also been observed, the cells being, in this case, best named zoospores (figs. 7 and 14); their release, however, could not be observed.

In some of the colonies examined, an amoeboid organism that fed on the cells of the alga, reminding use of the one described by Iyengar (1932) under the name *Vampyrella*, was found living in the mucilage (fig. 15).

Habitat

The physico-chemical parameters determined from the stream at Passadouro are given in table I.

Temp.	pH	Cond.	Miner.	Diss. O_2	% sat. O_2	COD_{Mn}	Alcal.	Cl^-
13.9	6.5	125	118	8.4	84	1.2	9.8	21

$N(NO_3^-)$	$N(NO_2^-)$	$N(NH_4^+)$	$P(PO_4^{3-})$	SiO_2	Na^+	K^+	Ca^{2+}	Mg^{2+}
1.44	det. lim.	0.030	0.062	3.6	12.1	1.3	3	1.8

Table I. - Parameters of the water in the stream at Passadouro (7/May).
det. lim.: determined value falls within the detection limit of the method used. Temp.: temperature in °C. Cond.: conductivity at 20°C in μ S. Miner.: mineralization in mg/l (evaluated from the conductivity at 20°C). % sat O_2: percentage of the maximum equilibrium O_2 concentration in pure water at the same temperature. COD_{Mn}: oxygen consumed during four hours at room temperature in acid medium (mg/l). Alcal.: alcalinity in mg/l of bicarbonate. All other values are in mg/l.

This stream is about 1.5 m wide and some 20 to 30 cm deep, and runs through agricultural fields. Not surprisingly, its margins are full of vegetation and its potential for primary production is high, as the determined values of nitrates and phosphates clearly show (see table I). Nevertheless, the water quality regarding the quantity and intensity of decomposition of dead organic matter is still good, as suggested by its transparency, the absence of foul smells, the relatively low value of COD_{Mn}, the sandy rather than muddy bottom, and the presence of *Batrachospermum*.

The ditch in Gerês occasionally receives some quantity of the effluent of a horse stable so that high levels of nitrogen compounds may sometimes be present in the water, although in general clean mountain water greatly reduces their concentration.

DISCUSSION

The authors providing descriptions of *Tetrasporidium* species can be divided into two groups: (1) those that encountered contractile vacuoles and include the genus in the Tetrasporales (Iyengar, 1932; Fott *et al.*, 1965; Sarma & Suryanarayana, 1969; Pandey *et al.*, 1980; Couté & Tracanna, 1981), and (2) one that found neither these structures nor the formation of motile cells and so includes it in the Chlorococcales (Nurul Islam, 1970). None of these authors expresses any doubt whether he is really in presence of *Tetrasporidium*, the identification at the genus level being based upon the general morphological characteristics of the thallus and not on cytological fine structure. The striking appearance of the organism at low power magnification, of which the several authors provide a figure, along with the absence of pseudocilia, seems in fact to justify this procedure, although it might appear unlikely that an organism would, in different occasions, show features that make it sometimes a member of Tetrasporales and other times one of Chlorococcales. The variability demonstrated by the organism studied in the present work suggests, however, that this may well be the case.

The nature of the organism may be understood as follows: (1) all of its cells are capable of converting themselves into swarmers under appropriate circumstances; (2) the swarmers typically have an eyespot and so have the cells that have progressed enough in their differentiation into motile cells; (3) when environmental conditions are inadequate for propagation by motile cells to occur the cells keep only their contractile vacuoles and do not form an eyespot or flagella; (4) the differentiation into swarmers can be arrested at any stage; (5) under given (possibly unfavourable) environmental conditions the cells can even loose the contractile vacuoles while keeping some degree of viability.

Since the vegetative cells of the organism show the capacity of converting themselves directly into flagellated cells with contractile vacuoles (and, in this case, also an eyespot) there can be no doubt that systematically it belongs to Tetrasporales, in which the family Palmellopsidaceae defines as in Ettl & Gärtner (1988) is appropriate.

For the determination of the species some further considerations are in order. Ettl & Gärtner (1988) consider three species in the genus, and provide a key for their identification. By following it one is confronted with the option between a parietal lobed chloroplast with the pyrenoid lying in a lateral position in the cell, and a cup-shaped chloroplast with a basal pyrenoid. The former option leads to *T. fottii* Couté et Tracanna while the latter follows to the distinction between *T. javanicum* and *T. lundii* Pandey *et al.*, the latter a species clearly inadequate for our material.

The species *T. fottii* was created by Couté & Tracanna (1981) for the organism described by Fott *et al.* (1965) from Czechoslovakia, based on the difference between the positions of the pyrenoid and of the nucleus: lateral and posterior, respectively, in Czechoslovakian material, while basal and central or anterior in French specimens. No difference is called upon between the structure of both types of chloroplasts and this is, in fact, the same: that of a parietal chloroplast with a conspicuous pyrenoid and a lobed margin. This is most evident in the drawings of Fott *et al.* (1965), and can also be seen in the drawings of Couté & Tracanna (1981), as well as in their figure 5 of plate II (a transmission electron micrograph) where a lobe is clearly visible on the left side of the cell.

The position of the nucleus is not a strong distinctive feature, as it is spatially limited by the other organelles, especially the chloroplast. Moreover, the organism being a member of Chlorophyceae, a strong connection between the nucleus and other internal structures of the cell (such as the membrane continuity with a chloroplast envelope that is common among the Chromophyta), that would tend to keep it in a fixed position, is not to be expected.

Since our material shows aspects that make it similar to both these "species", and until more is known about the variability within this genus, it is proposed that *T. fottii* Couté et Tracanna be considered synonymous with *T. javanicum* Möbius.

Although the organism seems to appear for only short periods in a place, after which it apparently disappears, along with the fact that the number of eyes on the lookout for natural novelties is nowadays considerably less than a few decades ago, its striking appearance makes it likely to be noticed after some time of occurrence in a region. These are, to the best of our knowledge, the furthermost western localities cited for *Tetrasporidium*, which might mean that it is expanding its area of distribution.

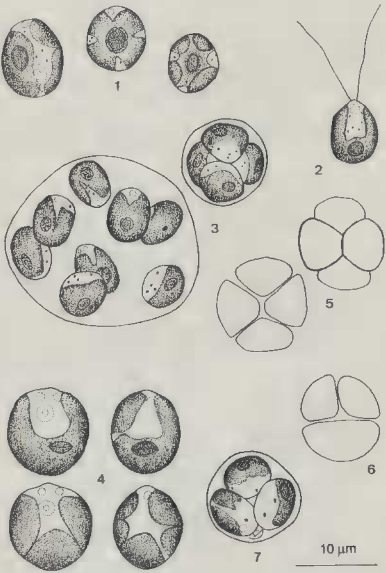
EXPLANATION OF PLATES

Plate I - 1. Vegetative cells. Note the position of the nucleus in the left cell. 2. Motile cells with two flagella of equal length. 3. Cell multiplication by the formation of 4 and 8 autospores. 4. Cells with apparently cup-shaped chloroplasts in optical section (top drawings) but showing their lobed margin near the surface (bottom drawings). 5. Formation of 4 autospores in one plane (left) and in two planes (right) (Schematic). 6. Formation of autospores by two not synchronous divisions: only one daughter cell has divided yet (Schematic). 7. Zoospores with motile flagella forming inside the parental cell wall; drawn after fig. 14.

Plate II - 8: *Tetrasporidium* in the stream at Passadouro attached to *Callitriche stagnalis* and to a plastic tube. 9. Net-like appearance under low power magnification; in B the edge of the thallus can be seen. Scale bar: 500 μm. 10. Disposition of the cells in the thallus. Scale bar: 50 μm.

Plate III - 11. Formation of autospores. Groups of 8 (left and center) and 4 (right) autospores can be seen. Note mother cell wall (arrowheads) and the chloroplast lobes (arrows). 12. groups of autospores still not separated (left and center) and 4 autospores already apart but still inside the parental cell wall (right); in B a different focusing plane of the center and right group of autospores can be seen. Note the contractile vacuoles (arrows). Scale bar: 20 μm (same scale throughout).

Plate IV - 13. Morphology of the chloroplast. A - cells showing seemingly cup-shaped chloroplasts although the pyrenoids are not perfectly basal; note the starch two-halved enclosure of the pyrenoid. B - Notice deeply lobed chloroplast (arrow). C - Large cell with apparently cup-shaped chloroplast; note the large contractile vacuole and the position of the pyrenoid. D - Note the clearly visible eyespot in two of the 4 autospores on the right (arrows). E - Note the lobes of the chloroplasts (arrows) and the position of the pyrenoids (arrowheads). 14. Zoospores; the arrow indicates the flagella (see also fig. 7). 15. Parasitic amoeboid organism. Scale bar: 20 μm (same scale throughout).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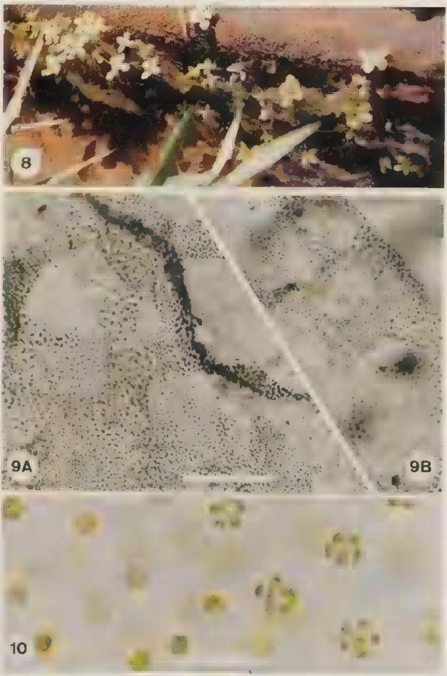
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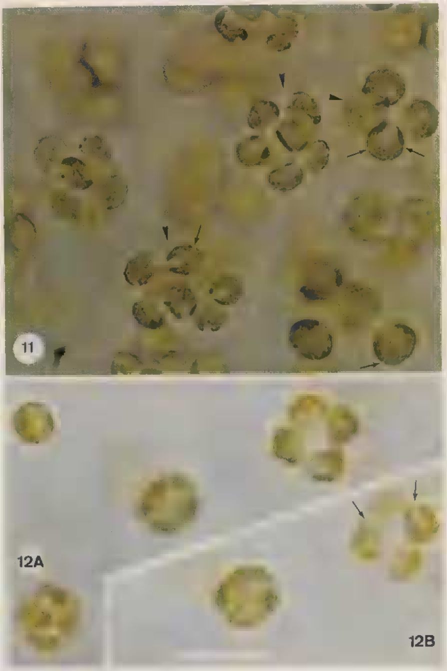
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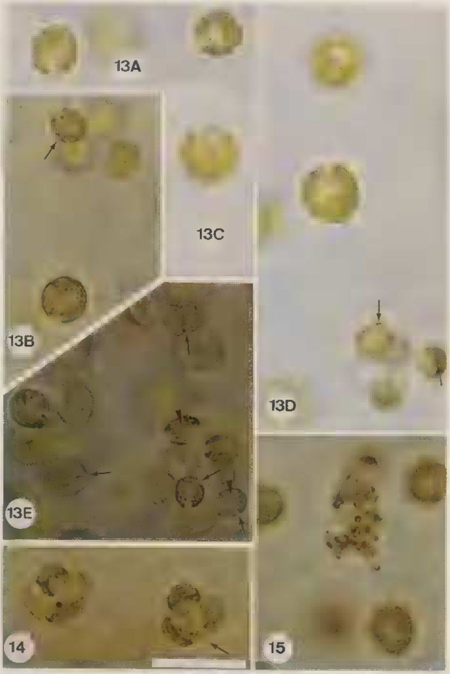
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